Amelioration of Clostridium difficile Infection in Mice by Dietary Supplementation With Indole-3-carbinol

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Objective: To determine the therapeutic effects of dietary supplementation on Clostridium difficile infection (CDI).

Background: With limited treatment options, the rise of C. difficile-associated disease has spurred on the search for novel therapies. Recent data define a role for the aryl hydrocarbon receptor (AHR) and diet-derived AHR ligands in mucosal immunity. We investigated the efficacy of indole-3-carbinol (I3C), a dietary supplement, and AHR precursor ligand in a murine model of CDI.

Methods: C57BL/6 (B6), AHR+/-, and AHR-/- mice were placed on either grain-based or semipurified diets with or without I3C before and during CDI. Mice were followed clinically for a minimum of 6 days or euthanized between days 0 and 4 of inoculation for analysis of the inflammatory response and microbiota.

Results: B6 mice fed an AHR ligand-deficient, semipurified diet have significantly increased disease severity (P<0.001) and mortality (P<0.001) compared with mice fed on diet containing I3C. The addition of I3C to the diet of AHR null mice had less of an impact than in AHR heterozygotes, although some protection was seen. Mice on semipurified I3C-diet had increased cecal Tregs, ILC3s, and γδ T cells and an increased neutrophilic response without infection or bacterial translocation compared with controls.

Conclusions: I3C is a powerful treatment to reduce impact of CDI in mice. The findings indicate I3C may be acting through both AHR-dependent and -independent mechanisms in this model. Dietary supplementation with I3C is a potential new therapy for prevention and amelioration of C. difficile disease.

Keywords: Aryl Hydrocarbon receptor, clostridium difficile, dietary supplement


*Clostridium difficile* infection (CDI) is a major health concern in the United States accounting for over 29,000 deaths per year and costing the healthcare system greater than $1 billion a year.† Disruption of the gut microbiome by the use of antibiotics is a major risk factor for *C. difficile*-associated disease (CDAD) in humans.‡ Other risk factors include age, acid suppression, immunosuppression, gastrointestinal surgery, and inflammatory bowel disease.† Primary treatment for acute infections is antibiotics. However, up to 20 percent of patients experience recurrence of CDI that may be resistant to subsequent treatment with antibiotics.‡ For these patients, novel therapies such as probiotics and fecal microbiota transplant are being tested.† The efficacy of probiotics in preventing infection has recently been brought into question,§ leaving the medical community with no options for disease prevention that are supported by data, other than minimizing antibiotic use and acid suppression. Fecal transplantation for recurrent/resistant disease has shown some promise,¶ but barriers to its adoption include difficulty in quality control, infectious issues, and dosing.

Although murine models have demonstrated the importance of microbiome integrity in minimizing CDI,† host immune cells, and their responses have also been shown to be critical. This includes the presence of group 3 innate lymphoid cells (ILC3s)¶ and the expression of IL-22.‡ The aryl hydrocarbon receptor (AHR) is critical for the development and maintenance of ILC3s and intraepithelial lymphocytes (IELs) of the intestine.¶ Both IELs and ILC3s are major sources of IL-22 in the gut¶ and the AHR is vital for the expression of IL-22 in these lymphocyte subsets and conventional T cells.¶ In addition to immune cell maintenance, dietary- and microbiome-derived AHR ligands have been shown to be effective in treating/ameliorating a different infectious disease (vaginal candidiasis) at mucosal surfaces in mice in an IL-22-dependent fashion.¶

Using this information, we hypothesized that the inclusion of I3C in the diet could reduce the severity of CDI. To test this hypothesis, we utilized a well-established murine model of CDI.¶ If this is indeed the case, such findings would suggest that the addition of a simple and safe dietary supplement begun shortly before planned antibiotics or other interventions that increase risk of CDI may have efficacy in reducing morbidity from this highly prevalent and severely debilitating disease.

**METHODS**

Mice

Approximately, 6- to 8-week old male C57BL/6j mice (B6) were purchased from Jackson Laboratory (Bar Harbor, ME). For some experiments, B6 mice were placed on either a grain-based chow or custom chow prepared to contain 1000 ppm I3C throughout the experiments. For those experiments where a diet low in AHR ligands was desired, mice were placed on either “semipurified diet” (AIN-76A Semi-Purified Diet; TestDiet, St. Louis, MO) or “I3C diet” (AIN-76A containing 1000 ppm I3C) for the remainder of the study. AHR heterozygotes (AHR+/-) mice, which have AHR expression equal to that of wildtype B6 mice, and AHR null (AHR-/-) mice on a
C57BL/6J background were bred and maintained under specific pathogen-free conditions. Animal experiments were carried out according to institutional guidelines. All procedures were approved by the University of Wisconsin School of Medicine and Public Health, Institutional Animal Care and Use Committee.

**Antibiotic Administration and Infection With C. difficile**

The model was adopted from the methods of Chen. Mice were monitored/scored twice daily for signs of clinical disease and weighed every morning. The experiment was terminated when all mice were euthanized based on the following criteria: clinical scores $\geq 13$, weight loss $\geq 20\%$, or at day 7 after inoculation. All scoring was done by investigators blinded to the experimental group. Cecal histology was examined and scored by a single blinded histopathologist as described in the supplement.

**Tissue and Stool Collection**

A modified version of a previously published protocol was used to prepare the cecum for histology and RT-PCR. Feces from mice were collected and stored at $-80\, ^\circ\mathrm{C}$ until further processing and microbiome analysis.

**Flow Cytometry**

Cecal cells were collected using a modified version of a previously published protocol. CEL and lamina propria (LM) cells were analyzed by flow cytometry.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). For evenly distributed continuous variables, Fisher exact t test was used to compare means. For unequally distributed data sets, a Welch’s correction was applied or a Mann-Whitney U test was applied. To analyze survival, groups were compared using Kaplan-Meier survival curves. $P < 0.05$ were considered significant. Additional detail to the methods can be found in the online supplemental materials, http://links.lww.com/SLA/B36.

**RESULTS**

**Dietary I3C Improves Outcome in CDI**

To investigate the role of the AHR in CDAD, AHR$^{-/-}$ mice, and AHR$^{-/-}$ littermate controls on standard chow were treated with ABx followed by inoculation with $10^5$ C. difficile spores. As shown in Figure 2A, AHR$^{-/-}$ mice had 100% survival at 5 days postinoculation. However, AHR$^{-/+}$ mice had reduced survival (67%; $P = 0.052$). Peak mean clinical score was day 3 postinoculation. Further measures of clinical disease severity, including percent weight loss and disease severity score (Fig. 2B, C) demonstrated that AHR$^{-/-}$ mice had significantly increased disease compared with AHR$^{-/+}$ mice. Thus, loss of AHR expression resulted in more severe C. difficile infection and disease. Similar to what was seen in wild-type B6 mice, AHR$^{-/-}$ mice on I3C-supplemented diet displayed increased survival and reduced weight loss and clinical score compared with mice on control diet (Fig. 2D–F). The significant protection in survival seen in I3C-supplemented mice was lost in littermate AHR$^{-/-}$ mice, although they did trend towards nonsignificant improvement in survival (Fig. 2G). Significant differences between the semipurified diet AHR$^{-/-}$ mice versus the I3C-diet AHR$^{-/-}$ mice were found for both weight loss and clinical score (Fig. 2H, I) at some time points. The peak mean clinical score for control diet AHR$^{-/-}$ mice was day 5 versus day 7 for I3C-fed AHR$^{-/-}$ mice. Mortality was also delayed in I3C-fed AHR$^{-/-}$ mice. Thus, CDAD was delayed by I3C supplementation in AHR$^{-/-}$ mice, and had less amelioration than seen in wild-type mice. These data suggest that some of the I3C-protective effects require the AHR, but I3C also has protective effects in this model that do not require the AHR.

**Alteration in Mucosal Immune Cells in Mice Fed Semipurified Diet**

Mechanistic experiments focused on mice maintained on semipurified diet, as these mice had the poorest outcome with CDI and received the most benefit from dietary supplementation. As anticipated based on previous findings, I3C-fed mice had significantly increased cecal Cyp1a1 mRNA expression compared with mice on semipurified diet suggesting greater AHR activation (Fig. 3A). Importantly, Cyp1a1 levels were significantly elevated both pre- and postantibiotic exposure in I3C-fed mice, suggesting that the majority of AHR activation was because of diet-derived ligands as opposed to microbial-derived AHR ligands. Mice on I3C diet also had higher levels of FoxP3 mRNA both before and after CDI (Fig. 3B). This finding was confirmed by flow cytometric measurement of FoxP3$^+$ CD4$^+$ T cells from the cecal lamina propria (CLP) on the day of C. difficile inoculation (Fig. 3C, D). At the same time point, we also found a higher proportion of ILC3s in the CLP of I3C-fed mice (Fig. 3C, E). In addition, upon evaluation of cecal IELs, mice on semipurified diet were found to have significantly less γδ T cells than their I3C-fed counterparts (Fig. 3C, F). Together, the decreased numbers of these gut immune cells in mice on semipurified diet may leave the host more susceptible to infection and increase the risk of an overly robust and pathologic immune response.

We did consider the possibility that the different diets could lead to differences in the microbiome that could account for differences in survival after CDI. As seen in Supplemental Figure 1, http://links.lww.com/SLA/B36 the difference in loss of diversity between the semi-purified diet and I3C-supplemented diet (before or after antibiotics) is small, and likely does not account for the dramatic improvement in survival with I3C-supplementation.

**Dietary Supplementation With I3C Increases Neutrophil Response But Reduces Inflammation**

Despite significant differences in the clinical course of CDAD in diet– versus diet + I3C-fed mice, no difference in colony-forming units of C. difficile in the cecum of mice at day 2 and day 4 was found between the 2 dietary groups (Fig. 4A). As studies have demonstrated...
both a protective and pathologic role for the host immune system in response to infection, and bacterial clearance of pathogens other than *C. difficile* by immune cells may be important for survival in this model.\textsuperscript{13} The neutrophil response to CDI was examined in both sets of mice. Before CDI, mice on semipurified and I3C diet had similar levels of cLP neutrophils (Fig. 4B, C). However, by day 3 of CDI, I3C mice had significantly more cLP neutrophils than semipurified diet mice. Despite this increase in neutrophils, I3C mice did not show more cecal inflammation than semipurified diet mice when analyzed by histopathology (Figure 4 D, E). To further examine the immune response to CDI, cytokine production in the cecum was examined. We hypothesized that IL-22 in particular would be more significantly elevated in mice on I3C supplementation compared with those without I3C supplementation, given the close association of this cytokine with AHR activation and its known protective effect in colitis. We did find a significant elevation of IL-22 in the I3C-supplemented group on day 4 after infection, as predicted. However, a similar elevation of IL-22 was seen in mice on semipurified diet without I3C-supplementation when analyzed on day 4 after infection (Fig. 4F, G). Differences in IL-17 and IFN-γ were also examined and were not different between mice on the 2 diets (Fig. 4 F).

**Addition of I3C to the Diet Decreases Bacterial Translocation During CDI**

Bacterial translocation has been identified as a main contributor to disease severity in mice deficient in IL-22 or in neutrophil influx.\textsuperscript{13,27} To examine whether supplementation of diet with I3C maintains epithelial integrity during CDI and decreases bacterial translocation, tissue was harvested from four sites from semipurified and I3C-fed mice on day 4 post-CDI and viable bacterial counts were determined using standard methods.\textsuperscript{13} Although both groups exhibited measurable bacterial loads in liver and lung with little to no
measureable bacterial loads in kidney, I3C-fed mice had significantly less bacterial counts in spleens and a trend towards lower counts in lungs compared with control mice (Fig. 5 A–D). The possibility that reduction of translocation is a mechanism of protection in this model was tested by treating mice during CDI with the oral antibiotic ciprofloxacin, an antibiotic with no action against this strain of \textit{C. difficile} but with efficacy against many other translocating bacteria. 

When mice on semipurified diet were treated with ciprofloxacin beginning after inoculation, survival, weight loss, and clinical score were all dramatically improved (Fig. 5 E–G). Together, these findings suggest that dietary I3C may contribute to prevention of bacterial translocation after CDI.

**DISCUSSION**

In this study, we have demonstrated that the addition of I3C to either grain-based standard chow or phytochemical-free semipurified diet reduced the morbidity and mortality associated with CDI in a
murine model. This finding has important clinical ramifications as the addition of I3C to the diet of patients scheduled for future procedures that include periprocedural antibiotics, or to any patient population known to be at increased risk for CDAD, may have efficacy in reducing the incidence and/or severity of CDAD. Furthermore, the semipurified diet may better represent the population in humans that fare the worst with CDI, many of who are malnourished or NPO (nil per os), whereas in the hospital. In addition, the potential exists that for patients who have previously had CDI, the addition of I3C to their diet might reduce the incidence of recurrence. The impact of these findings is strengthened by the fact that I3C is a safe dietary supplement that has already gone through clinical trials in humans, and is present in food, and could be added to the diet of patients at risk without the need for extensive testing or approval.

I3C is a weak AHR ligand and a metabolite of glucoraphanin, a natural product derived from members of the cabbage family. After consumption, I3C is converted by acid-mediated catalysis into various byproducts including the strong AHR ligands diindolylmethane (DIM) and indole[3,2-b] carbazole (ICZ). To test whether the effects on CDAD seen in mice fed I3C were mediated through the AHR, we utilized AHR null mice plus their heterozygous AHR<sup>+</sup>/<sup>−</sup> littermates. Mice lacking the AHR developed worse CDAD, as measured by weight loss, clinical score, and mortality compared with littermate controls. This finding was predicted given the effects of I3C on Cyp1a1 and FoxP3 expression as measured by RT-PCR in semipurified diet mice at day 0 demonstrating frequency of cLP Tregs (Live/CD4<sup>+</sup>/CD11b<sup>−</sup>/Ly6G<sup>−</sup>/CD3<sup>−</sup>/FoxP3<sup>+</sup>); ILC3s (Live/CD45<sup>+</sup>/CD11b<sup>−</sup>/Ly6G<sup>−</sup>/CD3<sup>−</sup>/CD4<sup>−</sup>/RORγt<sup>+</sup>); E), and number of IEL yδ T cells (Live/CD45<sup>+</sup>/CD11b<sup>−</sup>/Ly6G<sup>−</sup>/TCRβ<sup>−</sup>/CD3<sup>−</sup>/TCRγδ<sup>+</sup>); F). All data are representative of n = 4 per group, with error bars showing SEM, repeated in 2 independent experiments. *P < 0.05; **P < 0.01. Two-tailed Student t test or Mann-Whitney U test used for comparing averages.
FIGURE 4. Mice fed I3C supplemented diet display increased neutrophil response to *C. difficile* without increased cecal inflammation. A, Cecal *C. difficile* CFU/g feces in diet and diet + I3C mice at day 2 and 4 (n = 4 per group). Limit of detection indicated by dash-line. Determination of the presence or absence of *C. difficile* CFU for each day determined by $\chi^2$-analysis. B and C, Representative flow plots and % of cLP neutrophils per CD45$^+$ cells on day 0 (n = 4 per group) and day 3 (n = 8 per group) after inoculation in diet and diet + I3C mice. D and E, Representative histology at 100x and 400x in semipurified diet and I3C mice at day 3 (arrows depict neutrophils) and corresponding histopathological scoring (n = 10 per group). F, Relative IL-22, IL-17, and IFN-γ expression at day 0 and 4 as measured by RT-PCR in semipurified diet and diet + I3C mice normalized to β-actin (n = 4–10 per group). G, Measurement of total IL-22 and IL-17A protein content per cecum by ELISA.
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VPI 10463 mice may lessen the utility of mucosal immune cells that has been described in AHR-/- mice.\(^\text{15}\) When mice were placed on either semipurified diet or I3C-supplemented diet, I3C-fed AHR heterozygous mice demonstrated improved clinical outcomes as measured by increased survival, and reduced CDAD, similar to that seen in the wild-type B6 mice. I3C-fed AHR-/- mice did not have significantly improved survival compared with nulls on semipurified diet, but they did show reduced clinical scores and weight loss with supplementation, suggesting that I3C may be acting through AHR-dependent and independent mechanisms. Although it is not surprising that I3C might have mechanisms of protection that are AHR-independent, the global AHR null is imperfect as a control for the role of the AHR in gut disease. AHR null mice are known to have a patent ductus venosus.\(^\text{33}\) This is relevant to a colitis model, as the patent ductus prevents gut-derived blood from interacting with immune cells and detoxifying properties of the liver, and could have an effect on the microbiome of these mice as well. This phenotypic change in AHR-/- mice may lessen the utility of the global null as a control for the role of the AHR in gut disease in mice. Future studies using cell-specific AHR deletion will better define the role of the AHR in our model. Regardless, our data would suggest that the effects include AHR dependent and independent mechanisms.

Our studies examining the mechanism(s) of I3C-mediated control of CDAD utilized the semipurified phytochemical-free diet with and without I3C. The most striking difference in the phenotype of mice on these diets was found in the gut immune cells. Specifically, mice supplemented with I3C had more Tregs, ILC3s, and γδ T cells in the gut than mice on ligand-deficient diet. The increase in Tregs may limit the immune response to \textit{C. difficile} at the site of infection and decrease host-derived pathology. Indeed, in other models of colitis, Tregs have played a critical role in preventing disease.\(^\text{34,35}\) ILC3s play a crucial role in host resistance to intestinal pathogens, are important sources of IL-22 in the gut, and are implicated in preventing CDAD in mice.\(^\text{12}\) Finally, although γδ T cells have not been specifically examined in \textit{C. difficile}, they are known to play a critical role in maintaining gut immunity through the production of IL-22, IL-17, and other protective cytokines.\(^\text{18}\) and loss of these cells leads to bacterial overgrowth.\(^\text{15}\)

Our data support that the protection from morbidity or mortality in CDI was secondary to a modulation of the immune response, as opposed to a reduction of \textit{C. difficile} proliferation and growth. We did consider the possibility that the diet deficient of AHR ligands led to more dysbiosis and left these mice more susceptible to CDAD secondary to microbiome changes. There were some differences in the bacterial profile in I3C-supplemented mice, which may play some role in the protection from CDAD seen in this model. In general, mice on both diets had more similarities than differences, and the mild changes seen are unlikely to fully explain the dramatic improvement in outcome with I3C supplementation. A previous study did identify that oral supplementation of the gut enzyme intestinal alkaline phosphatase (IAP) during antibiotic treatment protects mice from CDAD, possibly by preserving the normal intestinal flora.\(^\text{36}\) As this did not appear to be the mechanism of protection in I3C supplementation, it may be interesting to consider supplementation of both IAP and I3C for more robust protection in future studies.

In response to infection, mice supplemented with AHR ligands demonstrated early influx of cecal neutrophils without an increase in inflammation or decrease in total number of \textit{C. difficile} CFU. We hypothesize that this increase in recruitment of neutrophils may be mediated in part by the increase in both ILC3s and γδ T cells and their associated cytokines.\(^\text{37}\) Given the finding that the increased neutrophil infiltrate does not increase the total amount of inflammation seen on histology, and the fact that the total number of \textit{C. difficile} bacteria is not different between the diets despite dramatically different survival, we hypothesize that the neutrophils prevent overwhelming translocation of commensal gut bacteria that would cause the host to die from sepsis. Indeed, I3C-supplemented mice have a reduced bacterial presence in the spleen compared with control mice. Although the identity of the translocating bacteria remains to be determined, the importance of bacterial translocation is supported by the finding that ligand-deficient mice can be rescued...
from *C. difficile* disease by treatment with ciprofloxacin. As ciprofloxacin has no efficacy against this strain of *C. difficile* but is effective against other commensal bacteria, the translocating bacteria are not likely to be *C. difficile*.

Current treatment strategies for CDI are directed at patients who have already presented with symptoms, an approach that can be too late to reverse severe disease. Some practitioners are attempting to manipulate the host microbiome to prevent CDAD, primarily with probiotic supplementation, but results have been disappointing. Although the role of dietary AHR ligands in preventing CDAD in humans has not yet been studied, a number of risk factors for CDAD have been identified. Patients on tube feeding carry an increased rate of CDAD, and whereas the mechanism of this is unclear, it is known that commonly used elemental feedings are absorbed in the small intestine and do not reach the colon. These patients may be at risk for a similar lack of AHR ligands present in their colon as seen in mice on ligand-deficient diet. Furthermore, those most at risk for developing CDAD are elderly, moribund, institutionalized, and antibiotic-exposed patients. These patients are at high risk for having decreased exposure to AHR ligands in their gut from both dietary and microbial sources, potentially leading to a poorly maintained gut immune system. Our study offers powerful new insights in the prevention of CDAD, where simple dietary supplementation of readily available AHR ligands could have profound impacts on rates and severity of CDAD in high-risk patients. In addition, given that mice on regular diet also saw protection from CDAD with supplementation, it would make sense to consider dietary supplementation as little as two weeks before an elective treatment that requires antibiotics, which is known to increase the risk of CDAD. This would apply to any patient that is scheduled to undergo a procedure or operation that requires perioperative antibiotics, scenarios where CDI can be more expensive, morbidity, and even life threatening than the procedure itself. Another area of intervention may be patients at risk for recurrent disease, which can affect as many as 25% of patients and is particularly morbid for those afflicted. Future experiments will directly test the efficacy of oral supplementation in a model of disease recurrence. In summary, this report represents the first strong evidence that we are aware of that simple addition of a safe oral supplement already found in our diets may maintain gut integrity and minimize the onset and morbidity of a severely morbid iatrogenic disease that has truly become an epidemic in our hospitalized patients. Given the burden of CDAD, dietary supplementation represents an exciting new treatment paradigm that could have a major impact in healthcare in the imminent future.

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**REFERENCES**


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